Multi-Laboratory Comparison of Three Heparin–Mn2+ Precipitation Procedures for Estimating Cholesterol in High-Density Lipoprotein

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Plasma high-density lipoprotein is commonly estimated by measuring the cholesterol remaining in plasma supernatant solutions after other lipoproteins, which contain apolipoprotein B, are precipitated with heparin and Mn2+. The method (method I) now in use by the Lipid Research Clinics, in which Mn2+ is at 46 mmol/liter final concentration, is reasonably accurate, but precipitation and sedimentation of lipoproteins other than high-density lipoproteins is often incomplete. We evaluated two modifications of method I. In method II, the Mn2+ concentration was doubled; the second modification (method III) included the increased Mn2+ concentration in a combined heparin Mn2+ reagent, decreased sample volume (2 ml), and a shorter incubation time (10 min at room temperature). The percentages of samples with turbid supernates (i.e., incomplete sedimentation) by methods I, II, and III were 9, 3, and 2%, respectively. Among non-turbid supernates, the percentages of samples containing measurable apolipoprotein B (incomplete precipitation) were 79, 19, and 16%, respectively. We conclude that method III is the most convenient and accurate of the three procedures.

High-density lipoprotein (HDL)³ concentrations have recently been shown to be strongly and inversely correlated with coronary heart disease incidence (1–3). Not only do myocardial infarction survivors have lower HDL concentrations in their plasma than do controls matched for cholesterol and triglyceride (4), but high HDL cholesterol values are associated with both increased longevity and low incidence of morbidity and mortality from myocardial infarction (5). Thus, with the new awareness of the importance of HDL as a negative risk factor for coronary heart disease, the demand for such measurements has dramatically increased.

HDL is generally measured by measuring the cholesterol in it (6). A common procedure for doing so involves precipitating the non-HDL (apolipoprotein B-associated lipoproteins) with sodium heparin at 1.2 to 2.0 g/liter and MnCl₂ at 46 mmol/liter final concentrations (7–9) and measurement of cholesterol in the supernatant solution. This method appears to be reasonably specific and is not often subject to large errors (10). However, it is less than ideal for estimating HDL-cholesterol in plasma, because about 10% of the plasma samples so precipitated have supernates with obvious turbidity, indicating incomplete sedimentation. Furthermore, about half of the samples with non-turbid supernates still contain apoB-associated cholesterol (mean, 25 mg/liter), leading to slight overestimation of HDL cholesterol (11). Recently, Warnick and Albers showed that a twofold increase in Mn²⁺ concentration, to 92 mmol Mn²⁺/liter, improved precipitation of the apoB-associated lipoproteins without substantial precipitation of HDL and better sedimented the apoB-associated lipoproteins from hypertriglyceridemic plasma (11). They recommended a more nearly accurate and more convenient modified version of the heparin–Mn2+ precipitation technique; namely, a twofold increase in the Mn²⁺ reagent concentration, addition of heparin–Mn²⁺ as a combined reagent, a decrease in sample volume from 3 to 2 ml, and a briefer sample incubation time, 10 instead of 30 min at room temperature rather than 4 °C. They also demonstrated that supernates that were turbid after low-speed centrifugation can be cleared by centrifugation at 12 000 x g for 10 min.

Here, we report results of a collaborative study by eight laboratories of the Lipid Research Clinics Program, to evaluate these changes in the heparin Mn²⁺ precipitation procedure. The modified procedure is shown to be both more specific and more convenient.

Materials and Methods

Samples. Plasma was sampled from subjects participating in population, genetic, and coronary prevention studies as well as from referral patients and healthy volunteers. Blood from the antecubital vein of subjects who had fasted 12–14 h was drawn into evacuated blood-collection tubes (Vacutainer Tubes) containing disodium ethylenediaminetetraacetate, 1.5 g/liter final concentration, according to Lipid Research Clinic protocol (9). Immediately after thorough mixing, the samples were cooled at 4 °C and centrifuged within 2 h. Samples were selected to obtain a range of lipid concentrations. Precipitation with heparin–Mn²⁺ was done within five days of sample collection. Samples were stored at 4 °C in the interim.

Reagents. Solutions of MnCl₂·4 H2O, 1.00, 2.00, and 1.06 mol/liter (197.9, 395.8, and 209.8 g/liter) were prepared at each

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Nonstandard abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; and apoB, apolipoprotein B.

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participating laboratory. A sample of each was submitted in a sealed vial to the Seattle laboratory for verification of Mn2+ concentration (12). All solutions were prepared by diluting commercial heparin preparations (Riker Lipo Hepin, 40 000 USP units/ml, 264–278 g/liter; Upjohn’s heparin, 10 000 units/ml, 69 g/liter; Organon’s heparin, 10 000 units/ml, 65 g/liter) to 5000 units/ml with 0.15 mol/liter NaCl solution. A combined heparin–Mn2+ solution was prepared by adding 0.6 ml of 40 000 USP units/ml heparin solution to 10.0 ml of 1.06 mol/liter MnCl2 solution. Laboratories using stock heparin of lower initial concentration adjusted the volumes in preparing this reagent to provide equivalent units of heparin/ml final solution.

Procedure. Portions of each plasma sample were precipitated with heparin and Mn2+:  

(Method I) 3.0 ml of plasma was precipitated by adding 0.12 ml of heparin (5000 USP units/ml), mixed, and 0.15 ml of 1 mol/liter Mn2+ (final concentration 46 mmol/liter) was added, mixed, incubated for 30 min at 4 °C, then centrifuged 1500 × g for 30 min at 4 °C (9).  

(Method II) 3.0 ml of plasma was precipitated exactly as above except that 2 mol/liter Mn2+ (92 mmol Mn2+/liter final concentration) was used.  

(Method III) 2.0 ml of plasma was precipitated with 0.20 ml of the combined heparin–Mn2+ working solution (92 mmol of Mn2+ per liter, final concentration) (11). Samples were mixed thoroughly and allowed to stand 10 min at room temperature before centrifugation at 1500 × g for 30 min. The nature of the supernatant solution was noted—i.e., clear, slightly turbid, turbid, very turbid, or clear with lipoprotein aggregate layer over the solution. In some samples, tiny pellets of manganese oxide were noted on the surface, but this did not represent incomplete precipitation. Any turbidity in the solution indicates incomplete removal of apoB-associated lipoproteins. Most samples produced a clear supernatant solution above the sedimented lipoprotein complex. The clear supernatant solutions were removed by pipetting. Turbid supernatates were immediately recentrifuged at 12000 × g for 10 min in the 40.3 rotor (Beckman Instruments Inc., Fullerton, Calif. 92634). The complexed lipoproteins generally formed a layer over the clear solution. A sample was obtained with a Pasteur pipet (pulled to a fine tip in a flame) by penetrating the aggregate layer. Alternatively, the samples were aspirated with a needle and syringe through the side of the tube. An aliquot of each clear heparin–Mn2+ supernatant solution was sealed in a vial and within two weeks of specimen collection shipped to the Seattle Laboratory, on ice. The extent of incomplete LDL–VLDL precipitation was estimated in each sample by measuring apolipoprotein B with a single radial immunodiffusion assay as outlined previously (11). ApoB values were multiplied by 1.6 to give apoB-associated cholesterol. The lower limit of sensitivity was approximately 5 mg of apoB-associated cholesterol per liter of plasma.

Cholesterol and triglyceride in the clear supernates were measured in each laboratory by continuous-flow analysis (AutoAnalyzer II; Technicon Corp., Tarrytown, N.Y. 10591) according to Lipid Research Clinic procedures, with use of the Liebermann–Burchard reagent for cholesterol and a variation of the Kessler and Lederer procedure for triglyceride (9). Carryover effects were minimized by analyzing each sample in duplicate, sequentially, and reporting only the second value. All fractions from a subject were analyzed sequentially in one AutoAnalyzer tray. However, the order of the sample pairs for each subject was randomized to reduce the systematic effects of instrument drift and scale expansion. The cholesterol data were submitted by each participating laboratory to the Seattle Clinic for compilation.

Statistical methods. The statistical significance of differences between HDL values was analyzed by use of the paired t-test (13).

Results

A total of 375 plasma samples were analyzed for HDL cholesterol by the three heparin–Mn2+ precipitation methods (Table I). Among laboratories there was considerable variation in the completeness of LDL–VLDL precipitation. Some 9% of the samples (32/375) that were precipitated at 46 mmol of Mn2+ per liter had obvious supernatant turbidity, indicating incomplete sedimentation of apoB-associated lipoproteins. Furthermore, of the samples with non-turbid supernates, 79% (270/343) contained apoB protein detectable by radial immunodiffusion, that is, >5 mg of apoB-associated cholesterol per liter of plasma. However, with precipitation at 92 mmol of Mn2+ per liter (method II) only 3% (12/375) of the supernates were turbid and 19% (68/363) had detectable apoB. Also, only 2% (9/375) of the plasma samples yielded turbid supernates when precipitated at 92 mmol of Mn2+ per liter by the modified procedure (method III) and 16% (58/366) of the non-turbid supernates had detectable apoB.

At each of the eight laboratories, precipitation by method II (92 mmol of Mn2+ per liter) gave slightly lower HDL cholesterol values than those obtained by method I (46 mmol of Mn2+ per liter), the overall mean difference being 25 mg/liter. At two laboratories, Seattle and Houston, HDL values obtained by method II were slightly but significantly (P < 0.01) higher than those obtained by method III. However, the other
Table 2. Total Cholesterol and ApoB-Associated Cholesterol in Non-Turbid Heparin Mn\(^{2+}\) Supernatant Solutions

<table>
<thead>
<tr>
<th>Location of clinic</th>
<th>Method I 46 mmol Mn(^{2+})/l</th>
<th>Method II 92 mmol Mn(^{2+})/l</th>
<th>Method III 92 mmol Mn(^{2+})/l</th>
<th>ApoB-associated cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Houston</td>
<td>445 ± 91</td>
<td>432 ± 84</td>
<td>417 ± 84</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Cincinnati</td>
<td>503 ± 175</td>
<td>482 ± 165</td>
<td>480 ± 169</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>Iowa City</td>
<td>487 ± 102</td>
<td>445 ± 96</td>
<td>448 ± 96</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>Minneapolis</td>
<td>479 ± 116</td>
<td>450 ± 110</td>
<td>448 ± 108</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>Seattle</td>
<td>527 ± 172</td>
<td>504 ± 162</td>
<td>495 ± 159</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>Palo Alto</td>
<td>542 ± 144</td>
<td>518 ± 144</td>
<td>516 ± 142</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>St. Louis</td>
<td>444 ± 219</td>
<td>440 ± 199</td>
<td>439 ± 195</td>
<td>24 ± 9</td>
</tr>
<tr>
<td>Toronto</td>
<td>496 ± 126</td>
<td>468 ± 117</td>
<td>463 ± 117</td>
<td>17 ± 14</td>
</tr>
<tr>
<td>Summary</td>
<td>498 ± 154</td>
<td>473 ± 146</td>
<td>467 ± 146</td>
<td>1</td>
</tr>
</tbody>
</table>

* Includes only samples for which clear supernates (infranates) of plasma were reported under all three precipitation conditions. Results expressed as mean ±SD in mg/liter.

six clinics found nearly identical HDL values by the two procedures. Overall, the mean difference in HDL values for non-turbid supernates between the two precipitation procedures at 92 mmol of Mn\(^{2+}\) liter was only 6 mg/liter.

Not only was the percentage of samples with detectable apoB in supernates much higher in plasma precipitated at 46 mmol Mn\(^{2+}\)/liter, as compared to 92 mmol/liter by method II (79% vs. 19%; Table 1), but also the value found for apoB-associated cholesterol in the non-turbid supernates was much higher than that found for plasma precipitated by method I, 17 mg/liter, as compared to 1 mg/liter (Table 2). Thus, the decrease in supernatant cholesterol by method II as compared to method I appears to be largely due to more complete precipitation of the apoB-associated lipoproteins at the higher Mn\(^{2+}\) concentration.

Plasma samples precipitated by method I that gave rise to turbid supernates generally had above-normal concentrations of triglycerides or cholesterol, or both (Figure 1). All nine of the plasma samples that had turbid supernates when precipitated by the 92 mmol Mn\(^{2+}\)/liter modified procedure had very high triglyceride (mean, 7520 mg/liter; range, 3680-15300 mg/liter) and cholesterol concentrations (mean, 3520; range, 2490-4180). Seven of these nine turbid supernatant solutions were recentrifuged at 12000 X g for 10 min. Five of the seven were essentially freed of apoB-associated lipoproteins by this centrifugation.

Plasma cholesterol and triglyceride concentrations, 2380 ± 620 mg/liter and 1630 ± 600 mg/liter, respectively, in nonturbid apoB-positive supernates tended to be higher than in plasma with nonturbid apoB-negative supernates, 2250 ± 660 and 1080 ± 600 mg/liter, respectively. The absolute decrease in supernatant cholesterol at 92 mmol of Mn\(^{2+}\) per liter (method II) compared to 46 mmol/liter (method I) was not highly correlated with either total triglyceride or LDL plus VLDL cholesterol. The correlation between the difference in HDL cholesterol values obtained at the two Mn\(^{2+}\) concentrations (HDL by method I minus HDL by method II), vs. the plasma triglyceride concentration, was 0.054. The correlation of the HDL difference vs. LDL plus VLDL cholesterol (total cholesterol minus HDL cholesterol by method II) was 0.032. Thus, the inability of 46 mmol of Mn\(^{2+}\) per liter to precipitate the apoB-associated lipoproteins completely does not appear to be primarily related to the plasma cholesterol or triglyceride levels.

**Discussion**

Our purpose was to compare among several laboratories some modifications of the heparin--Mn\(^{2+}\) technique for plasma HDL-cholesterol estimation. In harmony with previous observations, we conclude that the current method of using 1 mol of Mn\(^{2+}\) per liter (46 mmol/liter, final concentration) provides reasonably accurate quantitation if its limitations are recognized (10, 11). Some plasma samples, generally hyperlipemic, produced supernates that were turbid and grossly contaminated with the apoB-associated lipoproteins. This occurs when the density of the heparin--Mn\(^{2+}\)--lipoprotein complex is nearly that of the solution; sedimentation then is poor under the usual centrifugation conditions. Moreover, about 79% of the clear supernates contained measurable apoB-associated lipoproteins, as a result of incomplete precipitation. The mean apoB-associated cholesterol concentration level in clear supernates was 17 mg/liter. In terms of the mean HDL-cholesterol, 480 mg/liter, this overestimation is not a large error. However, in one laboratory all of the supernates contained apoB-associated cholesterol (mean, 49 mg/liter), suggesting that under some conditions overestimation of HDL may be considerable.

As reported previously, increasing the Mn\(^{2+}\) concentration
twofold to 92 mmol/liter considerably improved the precipitation of apoB-associated lipoproteins as well as improving sedimentations of precipitated lipoproteins in lipemic samples (11). The higher Mn2+ concentration reduced the apoB concentrations of the supernates and the proportion of supernates with detectable apoB. In addition, many samples with turbid supernates, which in the Lipid Research Clinics procedure necessitates reprecipitation at 46 mmol of Mn2+ per liter, sediment completely at the higher Mn2+ concentration.

A procedure in which this higher Mn2+ concentration is used, as well as other changes to improve the convenience, was evaluated (11). Heparin and Mn2+ were added as a combined reagent to 2.0 ml rather than 3.0 ml of plasma. Samples were incubated 10 min at room temperature rather than 30 min at 4 °C as in the LRC procedure. These changes decrease the sample requirement, pipetting steps, and time without significantly affecting results. HDL-cholesterol by the modified procedure (method III) was nearly equivalent to the HDL cholesterol by method II.

Although the proportion of samples with turbid supernates was lower, turbid supernates were still observed in 2 to 3% of the samples in this study when 92 mmol of Mn2+ per liter was used for precipitation, but the turbidity could be removed in most cases by subjecting the samples to 12 000 X g for 10 min (11). Thus the modified procedure has the following advantages over the present heparin-Mn2+ method: nearly complete removal of the apoB-associated lipoproteins for more accurate HDL quantitation; more complete sedimentation of precipitated lipoproteins decreases the number of samples requiring further manipulations to quantitate HDL; a smaller plasma volume is required; there are fewer manipulations in pipetting; and the procedure is more convenient because incubation and centrifugation can be performed at room temperature instead of at 4 °C.

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